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16. ABSTRACT

Rats were exposed to amosite asbestos and ferroactinolite fibers by intrapleural inoculation and intratracheal instillation. The ferroactinolite sample was found to be more carcinogenic in both exposures than the amosite sample on the basis of total fiber dose or fiber dose expressed for any size category of hypothetical greatest carcinogenic potency. Quantitative transmission electron microscope analysis of low-temperature ashed whole lung samples collected at different times following intratracheal instillation of fibers demonstrated that concentrations and sizes of fibers retained in rat lungs were greatly influenced by the relative ability of each mineral to undergo longitudinal splitting as a consequence of dissolution in vivo. Ferroactinolite fibers rapidly split to produce many thin fibers so that the number of ferroactinolite fibers retained in the lung 2 years after intratracheal instillation was four times greater than the number of fibers originally instilled. The number of short, thin ferroactinolite fibers retained (10-fold more than amosite) after in vivo splitting best explains the greater lung carcinogenicity of ferroactinolite compared to amosite.

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INTERPRETATION OF THE CARCINOGENICITY OF AMOSITE ASBESTOS AND FERROACTINOLITE ON THE BASIS OF RETAINED FIBER DOSE AND CHARACTERISTICS IN VIVO

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SUMMARY

Rats were exposed to amosite asbestos and ferroactinolite fibers by intrapleural inoculation and intratracheal instillation. The ferroactinolite sample was found to be more carcinogenic in both exposures than the amosite sample on the basis of total fiber dose or fiber dose expressed for any size category of hypothetical greatest carcinogenic potency. Quantitative transmission electron microscope analysis of low-temperature ashed whole lung samples collected at different times following intratracheal instillation of fibers demonstrated that concentrations and sizes of fibers retained in rat lungs were greatly influenced by the relative ability of each mineral to undergo longitudinal splitting as a consequence of dissolution in vivo. Ferroactinolite fibers rapidly split to produce many thin fibers so that the number of ferroactinolite fibers retained in the lung 2 years after intratracheal instillation was four times greater than the number of fibers originally instilled. The number of short, thin ferroactinolite fibers retained (10-fold more than amosite) after in vivo splitting best explains the greater lung carcinogenicity of ferroactinolite compared to amosite.

INTRODUCTION

A dose–response relationship for asbestos-induced malignant neoplasms in animals [1, 2] and man [3, 4] is generally recognized. Although animal exposures to different asbestos minerals have invariably been interpreted in terms of mass dose, currently popular theories for asbestos carcinogenicity are based on individual fiber characteristics such as size and shape. Pott [5] has suggested a hypothetical model for variable carcinogenic potency in relation to different fiber size categories and emphasized the importance of dose evaluation by measurement of fiber numbers in each size category. The term 'fiber' in this paper refers to mineral particles having

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parallel sides and a length to width (aspect) ratio ≥ 3. Transmission electron microscope analysis of exposure samples containing fibers, as received by the test animals, is required to obtain fiber dose information. Sample preparation procedures must insure that the fibers viewed on the electron microscope are representative of the original concentrations of fibers in each size category.

Evaluation of the carcinogenicity of fibers obtained from a sample of ferroactinolite, an amphibole mineral, relative to fibers of amosite, an amphibole asbestos with known human carcinogenicity, was accomplished by a detailed comparison of fiber doses used in rat intrapleural and intratracheal exposures [14]. The effective fiber dose which best explains differences in mineral fiber carcinogenic potencies may be quite different than the exposure dose. Retention of fibers in tissue over time is influenced by fiber deposition and clearance rates, movement of fibers in tissue, durability of the fibers in tissue, and changes in fiber morphology. Therefore, in this study the number and sizes of fibers retained in the lungs at different times following intratracheal instillation were determined for both ferroactinolite and amosite. We believe this represents the first successful attempt to link fiber carcinogenicity to quantitative measurements of fiber dose retained in the lungs during the lifetime of the test animals.

MATERIALS AND METHODS

The origin, mineral identity, and carcinogenicity of each mineral fiber test sample for intrapleural and intratracheal exposures to rats are described by Coffin et al. (submitted). Ferroactinolite and amosite fiber test samples were prepared for electron microscope analysis by suspending weighed amounts of the fine particles in filtered distilled water. Aliquots of these suspensions were filtered through 47 mm, 0.1 μm pore size Nuclepore* membrane filters. Electron microscope grids were prepared by direct transfer of carbon-coated filter pieces to Formvar-coated grids. The membrane filter and Formvar film were dissolved with chloroform by a wicking action leaving the fibers embedded in the thin carbon film suspended on the grid. This method is identical to the technique commonly used for analysis of particle concentrations in water samples [6].

Pairs of male Fischer-344 rats were killed at intervals of 1, 4, 12, and 24 months following 12 weekly intratracheal instillations of either 0.5 mg ferroactinolite or 0.25 mg amosite. Rats were also killed 1 day following single intratracheal instillations of either 0.5 mg ferroactinolite or 0.25 mg amosite and immediately following single intratracheal instillations of 5 mg of either test sample. The entire lower respiratory tract of each rat was removed and preserved in 10% buffered

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formalin. Electron microscope determination of the concentration and characteristics of fibers retained in the lung was accomplished by preparation of both lungs from each rat (trachea removed at the point where the bronchi enter each lung) as a single, homogenized sample. Each pair of lungs was freeze-dried and then low-temperature-ashed. The ash was suspended in filtered distilled water, aliquots were filtered through membrane filters, and electron microscope grids prepared with the technique previously described for the mineral fiber test samples.

Transmission electron microscopic (JEOL 100C) examination of randomly selected areas on several grids prepared for each sample involved the identification of mineral particles by morphology, selected area electron diffraction and energy dispersive X-ray spectroscopy. Particle size, shape, and mineral identification data were used for computer calculation of fiber size and shape distributions and concentrations. The incidence of large particles which are few in number but represent a large fraction of the sample mass was measured by examining larger areas of the grids for large particles only. The inclusion of this data created data sets which were based on observation of more than 1000 particles per sample. Five rat lung samples analyzed in duplicate produced an average coefficient of variation for fiber concentrations of 25%.

RESULTS

Preliminary electron microscope observation of ferroactinolite fibers obtained from rat lungs one year following intratracheal instillation indicated more fibers retained than expected from studies of the clearance of asbestos from lungs [7]. Many of the fibers present after one year appeared to be thinner than fibers in the exposure sample and chlorite plates common in the original exposure material were conspicuously absent. Larger ferroactinolite fibers frequently displayed numerous thin zones running parallel to the fiber long axis (Fig. 1). These zones are the apparent result of fiber dissolution in vivo. Occasional etch pits on fiber surfaces and leached zones perpendicular to the fiber's long axis were observed as described for acid leaching of mineral fibers [8]. Amosite fibers from lung tissue contained similar evidence of leaching but with fewer longitudinal thin zones.

Fiber concentrations (Table I) determined for lung samples from rats killed at different time intervals after intratracheal instillation of amosite and ferroactinolite confirmed the retention of large numbers of fibers throughout the lifetime of the animals. The four-fold increase in the number of ferroactinolite fibers 2 years after intratracheal instillation, $1.91 \cdot 10^9$ vs. $0.49 \cdot 10^9$ in the exposure, was unexpected. By contrast, the initial amosite dose was $1.71 \cdot 10^9$ fibers and the retained dose declined to $0.43 \cdot 10^9$ fibers 2 years later.

Mean fiber lengths, widths, and aspect ratios (Table I) describe changes in fiber morphology in vivo which parallel the changes in numbers of fibers retained in the lungs. Most striking is the dramatic 4-fold decrease in mean fiber width for

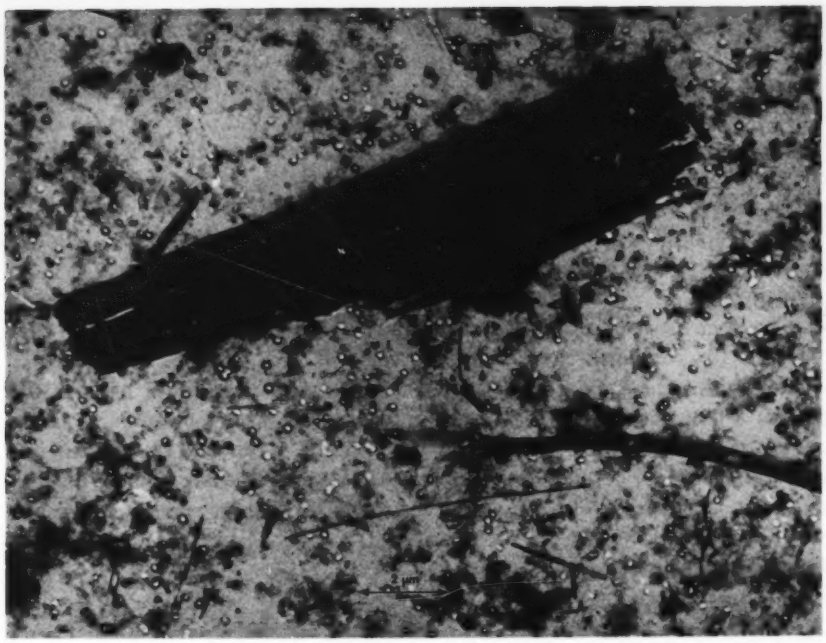

Fig. 1. Typical large fiber recovered from a rat lung 1 year after intratracheal instillation of ferroactinolite. Leaching in vivo produces longitudinal thin zones which act as foci for fiber splitting to produce very thin fibers also seen in this electron micrograph.

ferroactinolite fibers. This change cannot be explained by preferential lung clearance of thicker fibers since there is a 17-fold increase after 2 years in the numbers of ferroactinolite fibers with widths ≤ 0.1 μm. A doubling of the mean aspect ratio for amosite fibers after 2 years is a consequence of a slight decrease in fiber widths.

Both amosite and ferroactinolite fibers appears to undergo longitudinal splitting produced by fiber dissolution while residing in the lung. The ferroactinolite splitting reaction is more rapid and results in the formation of thinner and more numerous fibers than the amosite splitting reaction. The widths of the thin fibers produced correspond to the widths of longitudinal thin zones produced by fiber leaching on wider fibers (Fig. 1).

Fiber concentrations present at different intervals in the rat lungs following intratracheal instillation may be expressed in terms of different definitions for biologically active fibers. Evidence provided by Stanton [9] for greater carcinogenicity for fibers longer than 8 μm and thinner than 0.25 μm has received much attention [10–12]. Ferroactinolite and amosite concentrations for fibers of this

TABLE I

CHANGE IN MINERAL FIBER CONCENTRATIONS IN RAT LUNGS WITH TIME AFTER INTRATRACHEAL INSTILLATION

Time from end of intratracheal instillations	Fibers · 10^6/ mg of mineral instilled	Ferroactinolite			Fibers · 10^6/ mg of mineral instilled	Amosite		
		Mean length (μm)	Mean width (μm)	Mean aspect ratio		Mean length (μm)	Mean width (μm)	Mean aspect ratio
Exposure sample prior to instillation	81	3.18	0.41	9.0	570	3.44	0.29	11.8
0 days[a]	83	3.20	0.36	11.2	417	3.20	0.26	12.6
1 day[b]	107	2.86	0.37	10.8	585	2.60	0.24	10.9
1 month[c]	123	2.10	0.19	17.0	345	2.55	0.23	11.4
4 months[c]	262	2.00	0.17	22.3	393	3.02	0.22	15.7
12 months[c]	366	1.77	0.11	30.1	202	3.62	0.21	20.1
24 months[c]	313	1.64	0.10	25.4	141	3.61	0.20	18.4

[a] Single instillation (5 mg ferroactinolite and amosite).

[b] Single instillation (0.5 mg ferroactinolite, 0.25 mg amosite).

[c] 12 weekly instillations (total ferroactinolite = 6 mg, total amosite = 3 mg).

size category are plotted in Fig. 2. Lung concentrations of amosite fibers meeting Stanton's criterion for carcinogenic fibers decrease over the lifetime of the rats. The ferroactinolite sample has essentially no fibers meeting Stanton's criterion at the time of intratracheal instillation but fiber splitting produces $24.6 \cdot 10^6$ of such fibers in rat lungs 1 year after intratracheal instillation. The corresponding amosite dose at 1 year is $18.6 \cdot 10^6$ fibers. These numbers are calculated by multiplying the

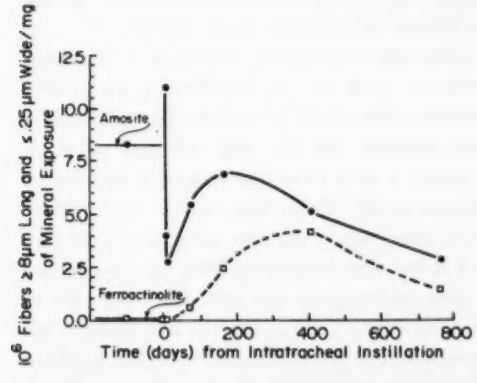

Fig. 2. Concentrations of long, thin fibers in rat lungs following intratracheal instillation. The number of fibers in the size range of length ≥ 8 μm and width ≤ 0.25 μm was determined by Stanton et al. [9] to best correlate with probability of pleural sarcoma following pleural implantation of fibers in rats.

intratracheal mass dose (6 mg for ferroactinolite and 3 mg for amosite) times the fiber concentrations reported in Fig. 2. At both 4 months and 2 years following the last intratracheal instillation of fibers the number of amosite fibers meeting Stanton's criterion slightly exceeds the corresponding number of ferroactinolite fibers.

DISCUSSION

The unexpected strong carcinogenicity of a ferroactinolite sample in comparison to amosite asbestos [14] cannot be explained on the basis of the fiber concentrations used in either the intrapleural or intratracheal exposures to rats. Expression of the fiber doses in terms of a currently popular theory which predicts greater carcinogenicity for long, thin fibers results in an even greater discrepancy between observed and predicted carcinogenicity. For example, if the dose is expressed as the number of fibers longer than 8 μm and thinner than 0.25 μm (Stanton's criterion [9]) and a linear dose response, no threshold relationship is assumed, the amosite sample would be predicted to induce 92 times more tumors in the intrapleural inoculation study and 46 times more tumors in the intratracheal instillation study.

Fiber concentrations retained in rat lungs (long after deposition and subsequent phagocytosis, muco-ciliary clearance, dissolution, splitting, lymphatic and hematogenous transport, and mechanical breakage) better explain the carcinogenic potency of the ferroactinolite. The actual time, during the sequence of events leading from exposure to observation of tumors, which provides the best point at which to measure the accumulated dose of fibers most indicative of a mineral's carcinogenicity is presently unknown. Since the time period from exposure to disease is much longer for man than for rodents, the kinetics of the various biological chemical and physical processes influencing fiber concentrations in vivo may dictate different relative carcinogenicities for mineral fibers in man.

The number of long, thin ferroactinolite fibers present in rat lungs 1 year after intratracheal instillations is approximately equal to the number of long, thin amosite fibers present after 1 year. Ferroactinolite, however, produced significantly more neoplastic lesions in the lung than amosite. In vivo fiber splitting of ferroactinolite produces many short, thin fibers. 1 year after intratracheal instillation there are 10 times more short, thin ferroactinolite fibers than short, thin amosite fibers residing in the lungs (Fig. 3). These data suggest that the number of very thin fibers of all lengths present in tissue is a better determinant of fiber carcinogenicity than the number of long fibers. This observation does not contradict the idea of increased carcinogenic potential of long, thin fibers, but supports and extends the concept of cumulative fiber carcinogenic potency proposed by Pott [5]. A recent statistical reanalysis of Stanton's data suggests that carcinogenicity may be a continuous function of aspect ratio and therefore short, thin fibers are carcinogenic [13].

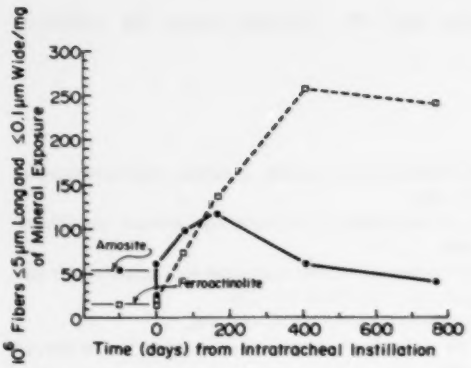

Fig. 3. Concentrations of short, thin fibers retained in rat lungs following intratracheal instillation. Fibers in this size range (length ≤ 5 μm and width ≤ 0.1 μm) are not observed by optical microscopic techniques used for occupational health monitoring of airborne asbestos.

Amosite produced 50% more pleural tumors than ferroactinolite following intrapleural inoculation [14], however, 600% more amosite fibers than ferroactinolite fibers were inoculated. Qualitative electron microscope examination of fibers removed from the pleural cavity months after inoculation indicates that fiber splitting occurs. The probable increase in the number of ferroactinolite fibers may be paralleled by an increase in the number of amosite fibers due to splitting. Very long amosite fibers, which tend to be much wider than shorter fibers, were placed in the pleural cavity and may have produced many very long, thin amosite fibers by splitting. The longest amosite fibers (100–600 μm) were not retained in rat lungs as a result of intratracheal instillation. Intrapleural inoculation studies could be more indicative of true carcinogenic potency if the size distributions of mineral fibers introduced are restricted to the size of fibers likely to be respireable and eventually transportable to the pleura.

We are examining chemical and structural determinants for in vivo fiber durability and dose alteration. We have observed the phenomenon of in vivo fiber splitting for other fibrous minerals such as crocidolite asbestos which produces fibers as thin as the ferroactinolite but at a slower rate. Chrysotile asbestos fiber bundles appear to rapidly dissociate to unit fibrils with diameters of approx. 300 Å and low durability in comparison to amphibole fibers. In addition to lack of durability, many mineral and synthetic fibers do not undergo splitting reactions and therefore are less likely to be human carcinogens when inhaled.

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